Journal of Andrology, Vol. 29, No. 4, July/August 2008

Characterizing the Glycocalyx of Poultry Spermatozoa: II. In Vitro Storage of Turkey Semen and Mobility Phenotype Affects the Carbohydrate Component of Sperm Membrane Glycoconjugates

JESÚS PELÁEZ AND JULIE A. LONG

From the Biotechnology and Germplasm Laboratory, Beltsville Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland.

ABSTRACT: The turkey sperm glycocalyx is known to contain residues of sialic acid, α-mannose/α-glucose, α- and β-galactose, α-fucose, α- and β-*N*-acetyl-galactosamine, monomers and dimers of *N*-acetyl-glucosamine, and *N*-acetyl-lactosamine. Potential changes in these carbohydrates during in vitro semen storage at 4°C were evaluated using males of both high- and low-sperm–mobility phenotypes. Changes in carbohydrate residues were quantified by flow cytometry analysis using a battery of 14 fluorescein isothiocyanate–labeled lectins in combination with control (sialylated) or neuraminidase-treated (nonsialylated) sperm. Sperm were evaluated at 0, 2, 4, 8, 12, and 24 hours of storage. For control sperm, 4 different patterns of lectin binding were observed over time: 1) increased mean fluorescence intensity (MnFI) at 2 hours (*Griffonia simplicifolia* lectin-I [GS-I]) and 8 hours (*Ricinus communis* lectin-I [RCA-I]) that remained elevated during storage; 2) increased MnFI at specific time points (*Limax flavus* lectin [LFA], 2 hours; *Artocarpus integrifolia* lectin [jacalin] and succinyl *Triticum vulgare* lectin [sWGA], 8 hours; *Galanthus nivalis* lectin [GNA], 12 hours) followed by decreasing MnFI during the remainder of the 24-hour storage period; 3) increased MnFI only at the 24-hour time point (*Lotus tetragonolobus* lectin [lotus] and *Arachis hypogaea* lectin [PNA]); and 4) no changes in MnFI during the 24-hour storage period (*Erythrina cristagalli* lectin [ECA], GS-II, *Pisum sativum* lectin [PSA], *Glycine max* lectin [SBA], and *Wisteria floribunda* lectin [WFA]). For nonsialylated sperm, increased binding of ECA, GS-II, SBA, and WFA was observed at variable time points; only *Canavalia ensiformis* lectin (Con A) and PSA remained unchanged during storage. Differences between mobility phenotypes existed for lectins Con A, GS-II, LFA, PSA, SBA, and sWGA, with sperm from low-mobility males exhibiting higher MnFI than high-mobility males throughout 24 hours of storage. We concluded that the observed increases in lectin binding during semen storage indicate an augmentation of nonsialylated terminal residues, which could alter sperm antigenicity and negatively impact fertility. Further, spermatozoa from low-mobility males may have higher antigenicity even before semen storage. Other possible functional implications are discussed.

Key words: Lectin, artificial insemination, glycoprotein, sperm physiology.

J Androl 2008;29:431–439

The commercial turkey industry relies exclusively on artificial insemination for fertile egg production. Current methodologies for storing turkey semen in vitro do not provide adequate fertility rates for commercial operations. Turkey semen stored at 4°C for up to 6 hours maintains acceptable fertility rates with weekly inseminations over the typical 6- to 7-month period of egg production; however, semen stored for longer periods of time results in a decline in fertility after 5 to 15 weeks of egg production (Etches, 1996; Donoghue and Wishart, 2000). The overall goal of our research is to understand why turkey spermatozoa lose functional competence during in vitro storage and develop methodologies that maintain sperm functionality for longer semen storage intervals (eg, 24–48 hours).

The sperm glycocalyx represents the primary interface between the sperm cell and its environment and is a critical component for sperm maturation, sperm transport, and sperm-egg interaction in mammals (Diekman, 2003). The functional significance of the poultry sperm glycocalyx is not well defined. It has been demonstrated that rooster spermatozoa acquire surface-associated glycoproteins during maturation within the male duct system that remain associated with spermatozoa in the female tract (Morris et al, 1987). Impaired fertility has been associated with alterations in the carbohydrate content of rooster spermatozoa (Froman and Engel, 1989). In particular, sialic acid is required for sperm passage through the hen's vagina (Steele and Wishart, 1996) and for sequestration in the hen's sperm storage

Supported in part by the USDA-ARS project "Analysis of Sperm Storage Mechanisms in Poultry" (CRIS project 1265-31000-83-00D) and the Ministerio de Educación y Ciencia (Spain). Mention of a trade name, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the USDA or imply its approval to the exclusion of other suitable products or vendors.

Correspondence to: Dr Julie A Long, USDA, ARS, ANRI, ABBL, BARC-East, Bldg 200, Room 120, Beltsville, MD 20705 (e-mail: julie.long@ars.usda.gov).

Received for publication September 20, 2007; accepted for publication March 17, 2008.

DOI: 10.2164/jandrol.107.004259

tubules (Froman and Thursam, 1994). *N*-acetyl-D-glucosamine, localized on the perivitelline layer of the chicken ovum, is necessary for sperm-egg interaction (Robertson et al, 2000). Although these data pertain to chickens, the possibility exists for similar functional implications of surface carbohydrates in turkey spermatozoa. Further, the loss and/or alteration of surface glycoproteins during semen storage also may impact the ability of turkey spermatozoa to traverse the hen's reproductive tract and recognize/bind to the ovum.

We recently characterized the carbohydrate composition of the turkey sperm glycocalyx in fresh semen from males with average-mobility semen by means of flow cytometry assessment of lectin binding (Peláez and Long, 2007). The glycocalyx of turkey spermatozoa is extensively sialylated and contains residues of α-mannose/α-glucose, α- and β-galactose, α-fucose, α- and β-*N*-acetyl-galactosamine, *N*-acetyl-lactosamine, and monomers and dimers of *N*-acetyl-glucosamine in variable amounts. Our aim here was to determine if these surface carbohydrates are altered during conventional in vitro semen storage at 4°C for 24 hours. Because the majority of terminal carbohydrate residues are masked by sialic acid, we used a neuraminidase treatment to detect differences in unmasked residues over time. Finally, we compared the carbohydrate composition of semen from high- and low-sperm–mobility phenotypes because males exhibiting high mobility indices have a competitive advantage with respect to paternity (Donoghue et al, 1998).

Materials and Methods

Animals

Male turkeys (Hybrid Grade Maker; Hybrid Turkeys, Ontario, Canada) were maintained at the Beltsville Agricultural Research Center poultry facilities under standard management practices, which included lighting conditions (14:10-hour light:dark cycle) for sperm production. Management complied with the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training (Allen, 2000). The care, treatment, housing, and use of turkeys for this project were approved by the Beltsville Area Animal Care and Use Committee.

Prior to initiation of the study, males were screened at 28, 32, and 40 weeks to determine their sperm mobility phenotypes. Sperm mobility is a quantitative trait of semen quality originally described in the fowl (Froman and McLean, 1998).

It has been well established that this parameter fits a normal distribution within a population of individual males, allowing the characterization of individual males with either low- or high-sperm mobility as classified by 1 standard deviation above or below the population mean. We assessed turkey sperm mobility according to the methods of King et al (2000) using a 1×10^9/mL sperm suspension, 6% (wt/vol) Accudenz solution (Accurate Chemical & Scientific Corp, Westbury, New York), and microreader photometer (IMV Microreader; IMV International Co, Minneapolis, Minnesota) to measure optical density at 597 nm after 1 minute of equilibration. After characterization, 6 males (3 high mobility and 3 low mobility) were randomly chosen for the study and assigned to the respective phenotype groups.

Semen Collection and Processing

Semen was collected manually (Burrows and Quinn, 1937) from each male twice per week, with a minimum 48-hour interval between the 2 collections. Immediately after collection, semen was pooled within the designated mobility group and diluted 1:1 with Beltsville Turkey Semen Extender II (Continental Plastics Corp, Delavan, Wisconsin). Aliquots of diluted semen from each mobility group were transferred to a 24-well culture plate (0.5–1 mL/well). The plate was covered loosely with aluminum foil, placed on an orbital shaker (125 rpm), and maintained at 4°C for 24 hours. Seminal plasma was removed from 150-μL aliquots of fresh (0 hours, nonstored) and stored (2, 4, 8, 12, or 24 hours) semen by centrifugation ($400 \times g$; 5 minutes). Sperm pellets were resuspended in the buffer appropriate for either lectin staining (control) or neuraminidase treatment followed by lectin staining (neuraminidase-treated [NT] samples).

Lectin Staining and Neuraminidase Treatment

Fourteen fluorescein isothiocyanate (FITC)–conjugated lectins (EY Laboratories Inc, San Mateo, California) were used to detect residues of 9 carbohydrate groups: 1) α-galactose (*Griffonia simplicifolia* lectin-I [GS-I], *Artocarpus integrifolia* lectin [jacalin]); 2) β-galactose (*Ricinus communis* lectin-I [RCA-I], *Arachis hypogaea* lectin [PNA]); 3) α-mannose/α-glucose (*Canavalia ensiformis* lectin [Con A], *Pisum sativum* lectin [PSA], *Galanthus nivalis* lectin [GNA]); 4) *N*-acetyl-glucosamine monomers (GS-II); 5) *N*-acetyl-glucosamine dimers (succinyl *Triticum vulgare* lectin (sWGA]); 6) α- and β-*N*-acetyl-galactosamine (*Glycine max* lectin [SBA], *Wisteria floribunda* lectin [WFA]); 7) α-fucose (*Lotus tetragonolobus* lectin [lotus]); 8) sialic acid (*Limax flavus* lectin [LFA]); and 9) *N*-acetyl-lactosamine (*Erythrina cristagalli* lectin [ECA]). The specificity of these lectins for the respective carbohydrate group was previously demonstrated (Peláez and Long, 2007). For all experiments, lectins were used at concentrations of 100 μg /mL in Tris buffer (TBS; 0.05 M Tris, 0.15 M NaCl [pH 7.6]). Lectins GS-I, GS-II, Con A, and PSA were prepared in TBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$.

Control sperm were resuspended in TBS to a concentration of 2.5×10^9 sperm/mL. A 2.5-μL aliquot of each sperm suspension was added to a 62.5-μL volume of lectin solution to yield a final concentration of 100×10^6 sperm/mL. Sperm-lectin mixtures were incubated protected from light for 30 minutes at room temperature. After incubation with lectins, samples were washed twice by centrifugation ($700 \times g$; 5 minutes), and sperm pellets were resuspended in the appropriate buffer (eg, TBS with or without cations). Ten

microliters of the final resuspension of each sample was diluted in 0.5 mL of the appropriate buffer and counterstained with 12 μM propidium iodide (PI; Molecular Probes, Eugene, Oregon) for a minimum of 5 minutes at room temperature.

Because the turkey sperm glycocalyx is extensively sialylated (Peláez and Long, 2007), neuraminidase treatment was used to detect changes in masked carbohydrates for all lectins (except the sialic acid–specific lectin LFA). After removal of seminal plasma, pelleted spermatozoa were resuspended in TBS (pH 6.0) to a final concentration of 1×10^9 sperm/mL and incubated for 30 minutes at 37°C with 1 IU neuraminidase (type V; *Clostridium perfringens*; Sigma-Aldrich, St Louis, Missouri)/10^9 sperm. After an initial centrifugation ($700 \times g$; 5 minutes), sperm were resuspended in TBS (pH 6.0) and washed once, followed by 2 centrifugations in TBS (pH 7.6). The final pellet was used immediately for lectin staining as described above.

Flow Cytometry Assessment of Lectin Binding

A Coulter Epics XL-MCL flow cytometer (Coulter Corp, Miami, Florida) equipped with a single 488-nm excitation source was used for all analyses. Forward and side scatter gating were used to select single sperm from clumps and debris. The fluorescence from FITC-stained and PI-stained spermatozoa was collected in FL1 (525-nm bandpass) and FL3 (620-nm bandpass) fluorescence detectors, respectively. Because cells with intact plasma membranes preclude lectins from binding to internal structures, only FITC fluorescence signals generated by PI-negative cells were considered in the analysis. The mean FITC fluorescence intensity/cell (MnFI) of the viable sperm population was recorded from the FL1 detector output as an indicator of lectin binding. The entire experiment (ie, semen collection, sample preparation, and flow cytometry assessment) was repeated 6 times with a minimum 1-week interval using the same semen donors.

Statistical Analysis

All analyses were conducted using STATISTICA software for Windows (version 7; StatSoft Inc, Tulsa, Oklahoma). $P < .05$ was considered significant. Data were first tested for differences between the MnFI values of control and NT samples to elucidate the lectins in which an effect of treatment on the fluorescence intensity exists. MnFI values obtained for each of the 14 lectins (n = 36 [6 time points × 6 replicates]) within mobility phenotype (high or low mobility) were subjected to separate 1-way analyses of variance (ANOVAs). If the test showed a significant effect of treatment, the rest of the analyses were conducted separately on either control or NT samples. If, on the contrary, a significant effect of treatment did not exist in any of the 2 mobility phenotypes, data from both control and NT samples were combined. Differences in the MnFI values obtained throughout the incubation period (time effect) and between semen mobility phenotypes (mobility phenotype effect) were tested using 2-way ANOVA. The model considered "mobility phenotype" as a fixed factor and "time" as a fixed factor with repeated measures. In the absence of significant interactions, when a significant effect of the mobility phenotype was observed, the effect of time was studied separately on each mobility sample (high- or low-mobility semen). Post hoc comparisons were made using the Student-Newman-Keuls test (Carrasco, 1995).

Results

A comparison of control and NT sperm revealed differences ($P < .05$) in the MnFI for most (10/13) lectins during semen storage (Table 1). Only RCA-I, PNA, and lotus exhibited similar MnFI for both control and NT spermatozoa throughout semen storage. Therefore, data from control and NT samples were combined for subsequent analyses of these lectins. For the high-mobility group, lectins PSA and WFA exhibited higher MnFI for NT spermatozoa than control spermatozoa ($P < .05$); whereas binding of these 2 lectins was similar ($P \geq .05$) between control and NT spermatozoa from the low-mobility phenotype males (Table 1).

The main effects of time and mobility phenotype on lectin binding are shown in Table 2. Significant changes ($P < .05$) in the MnFI occurred over time for 9 of the 14 lectins incubated with control spermatozoa. For lectins GS-II, WFA, and ECA, the time effect was observed only for NT spermatozoa ($P < .05$). The mobility phenotype effect was only significant ($P < .05$) for lectins Con A, PSA, and SBA. The interaction "time × mobility group" was not significant for any sample.

For most lectins, the changes detected in fluorescence intensity over time were related to elevated MnFI values. For control spermatozoa, higher MnFI ($P < .05$) was noted as early as 2 hours after storage (GS-I, 0.84 ± 0.08; LFA, 1.45 ± 0.03) compared with initial samples at 0 hours (GS-I, 0.65 ± 0.04; LFA, 1.17 ± 0.04). Also in control samples, the first significant MnFI increases for lectins sWGA and jacalin occurred at 8 hours of storage, whereas GNA binding increased at 12 hours compared with 0-hour samples ($P < .05$). For NT samples, most MnFI values were higher at the 8-hour time point compared with those of fresh semen, including lectins GS-II, ECA, SBA, and RCA ($P < .05$), whereas lectins GS-I and PNA had increased MnFI at the 12- and 24-hour time points, respectively ($P < .05$). The MnFI were not different between control and NT samples for lectins RCA or lotus, which increased at the 8- and 24-hour time points, respectively ($P < .05$). No changes in MnFI over time were apparent for lectins Con A or PSA ($P < .05$). Lectins SBA, WFA, and PNA also exhibited similar MnFI at each time point but only for control samples ($P < .05$).

Four basic patterns of lectin binding were observed over time when stored semen was compared with fresh semen: 1) increased MnFI ($P < .05$) between 2 and 8 hours (GS-I and RCA-I) that remained elevated for

Table 1. *The effect of neuraminidase treatment on mean fluorescence intensity of lectin binding (mean ± SE; n = 36) to spermatozoa from the high- and low-sperm–mobility phenotype males*[a]

Carbohydrate group	Lectin	High mobility		Low mobility	
		Control	Treated	Control	Treated
α-Galactose	GS-I	0.83 ± 0.03[b]	2.37 ± 0.16[c]	0.87 ± 0.04[b]	2.88 ± 0.27[c]
	Jacalin	0.97 ± 0.04[b]	1.28 ± 0.06[c]	1.18 ± 0.05[b]	1.53 ± 0.08[c]
β-Galactose	RCA-I	192.37 ± 8.42[b]	172.57 ± 16.65[b]	196.69 ± 7.23[b]	181.29 ± 13.72[b]
	PNA	0.67 ± 0.03[b]	0.68 ± 0.03[b]	0.69 ± 0.03[b]	0.73 ± 0.04[b]
α-Mannose/α-glucose	Con A	0.28 ± 0.01[b]	0.51 ± 0.02[c]	0.33 ± 0.01[b]	0.51 ± 0.02[c]
	PSA	0.69 ± 0.04[b]	0.83 ± 0.04[c]	1.01 ± 0.06[b]	1.13 ± 0.07[b]
	GNA	0.63 ± 0.02[b]	2.98 ± 0.08[c]	0.74 ± 0.03[b]	3.15 ± 0.08[c]
GlcNAc	GS-II	0.39 ± 0.01[b]	0.59 ± 0.03[c]	0.44 ± 0.02[b]	0.65 ± 0.04[c]
$GlcNAc_{(2)}$	sWGA	97.08 ± 5.52[b]	8.19 ± 0.48[c]	113.14 ± 6.56[b]	9.59 ± 0.77[c]
GalNAc	SBA	0.60 ± 0.03[b]	16.53 ± 0.64[c]	0.87 ± 0.05[b]	16.84 ± 0.71[c]
	WFA	0.60 ± 0.01[b]	0.67 ± 0.02[c]	0.64 ± 0.02[b]	0.70 ± 0.03[b]
α-Fucose	Lotus	0.44 ± 0.02[b]	0.47 ± 0.02[b]	0.44 ± 0.02[b]	0.49 ± 0.02[b]
LacNAc	ECA	0.59 ± 0.03[b]	24.97 ± 1.22[c]	0.75 ± 0.03[b]	27.09 ± 1.35[c]

Abbreviations: Con A, *Canavalia ensiformis* lectin; ECA, *Erythrina cristagalli* lectin; GlcNAc, *N*-acetyl-glucosamine; GalNAc, *N*-acetyl-galactosamine; GNA, *Galanthus nivalis* lectin; GS-I, *Griffonia simplicifolia* lectin-I; LacNAc, *N*-acetyl-lactosamine; PNA, *Arachis hypogaea* lectin; PSA, *Pisum sativum* lectin; RCA-I, *Ricinus communis* lectin-I; SBA, *Glycine max* lectin; sWGA, succinyl *Triticum vulgare* lectin; WFA, *Wisteria floribunda* lectin.

[a] Data for *Limax flavus* (LFA) lectin were not compared because neuraminidase treatment would remove the LFA-specific sialic acid carbohydrate from the sperm surface.

[b,c] Different superscripts indicate significant differences within rows and within mobility phenotypes ($P < .05$)

the remaining time points (Figure 1A); 2) incremental elevations in MnFI that peaked ($P < .05$) at specific time points (LFA, 2 hours; jacalin, 8 hours; GNA and sWGA, 12 hours) and subsequently decreased during the remaining time points (Figure 1B); 3) increased ($P < .05$) MnFI values only at the 24-hour time point (lotus and PNA; Figure 1C); and 4) no changes in MnFI ($P > .05$) throughout the 24-hour storage (ECA, GS-II, SBA, and WFA; Figure 1D). Seven lectins exhibited different binding patterns over time between control and NT spermatozoa. For NT samples, the MnFI values of lectins ECA and SBA followed pattern 1 (Figure 2), whereas lectin WFA exhibited pattern 2 and lectin PNA followed pattern 3 ($P < .05$) for NT spermatozoa. Conversely, the MnFI values of NT spermatozoa incubated with lectins GNA, jacalin, and sWGA did not change over time ($P > .05$).

Interestingly, although lectins Con A and PSA showed no changes in MnFI over time for either control or NT spermatozoa, the MnFI were consistently lower ($P < .05$) for semen from the high-mobility group when compared with semen from the low-mobility group (Figure 3). Similarly, although the lectin-binding pattern for sWGA followed pattern 2, the MnFI values were lower ($P < .05$) for the high-mobility group than the low-mobility group at each time point. The MnFI values for SBA differed ($P < .05$) between mobility groups for control spermatozoa but were similar ($P > .05$) for NT spermatozoa. Finally, it should be noted that each of the above differences or similarities in MnFI between the high and low mobility groups were observed in fresh semen (0 hours) prior to storage (Figure 3).

Discussion

The work presented here provides experimental evidence that the carbohydrate residues of membrane surface glycoconjugates in turkey spermatozoa undergo quantitative changes during 24-hour, low-temperature storage of semen. The magnitude and patterns of glycocalyx changes varied among sugar residue type, but the majority of changes in carbohydrate abundance occurred after 8 hours of semen storage. More importantly, males characterized as either high- or low-mobility phenotype exhibited striking differences in the content of certain carbohydrates and the changes associated with in vitro storage. These data are particularly relevant as physiological models for explaining the reduced fertility associated with in vitro stored turkey semen. The findings reported here may be of importance for a better understanding of the mechanisms by which cold storage affects sperm functionality, leading to a decrease in fertilizing capacity and ultimately the reproductive efficiency of the male.

The abundance of virtually all membrane surface carbohydrates was altered during the 24-hour storage period at 4°C. We focused first on the nontreated samples because those represented the physiological state of spermatozoa during in vitro semen storage. In general, 4 patterns of quantitative changes were

Table 2. *The effects of semen storage time and sperm mobility phenotype on the mean fluorescence intensity values of each lectin*

		P	
Carbohydrate group	Lectin	Time	Mobility phenotype
α-Galactose	GS-I	<.001	.61
	Jacalin	<.001	.08
β-Galactose	RCA-I[a]	<.001	.75
	PNA[a]	<.01	.49
α-Mannose/α-glucose	Con A[b]	<.001	<.05
	PSA[b]	.63	<.05
	GNA	<.05	.11
N-acetyl-glucosamine (monomers)	GS-II[c]	<.05	.48
N-acetyl-glucosamine (dimers)	sWGA	<.001	.30
α- and β-*N*-acetyl-galactosamine	SBA[b]	.05	<.01
	WFA[c]	<.01	.55
α-Fucose	Lotus[a]	<.05	.90
N-acetyl-lactosamine	ECA[c]	<.001	.55
Sialic acid	LFA	<.001	.59

Abbreviations: Con A, *Canavalia ensiformis* lectin; ECA, *Erythrina cristagalli* lectin; GNA, *Galanthus nivalis* lectin; GS-I, *Griffonia simplicifolia* lectin-I; PNA, *Arachis hypogaea* lectin; PSA, *Pisum sativum* lectin; RCA-I, *Ricinus communis* lectin-I; SBA, *Glycine max* lectin; sWGA, succinyl *Triticum vulgare* lectin; WFA, *Wisteria floribunda* lectin.

[a] Control and neuraminidase-treated data were combined because there were no significant differences ($P > .05$) between sperm treatments.

[b] Separate analyses were conducted for high- and low-mobility phenotypes and are reported within the text.

[c] Probability values represent only neuraminidase-treated samples; control samples were nonsignificant ($P > .05$) for time and mobility factors.

observed in nontreated spermatozoa: 1) increased MnFI at specific time points that remained elevated during storage, 2) increased MnFI at specific time points followed by decreasing MnFI during the remainder of the 24-hour storage period, 3) increased MnFI only at the 24-hour time point, and 4) no changes in MnFI during the 24-hour storage period. Changes in the abundance of *N*-acetyl-glucosamine and *N*-acetylgalactosamine residues over time were detected only after terminal sialic acid residues were removed with neuraminidase, and the timing of those changes corresponded mainly to pattern 2. The physiological significance of changes for those 2 carbohydrate groups could be considered less relevant than those observed for control sperm; however, it is possible that terminal sialic acid residues are cleaved or lost prior to sperm/egg binding in the female reproductive tract because carbohydrate moieties other than sialic acid are known to be required for gamete recognition (Robertson et al, 2000). Thus, alterations in the abundance of carbohydrate groups that are temporarily masked by sialic acid may be physiologically relevant. We recently demonstrated that localization of some carbohydrate groups to specific morphologic regions on turkey and chicken spermatozoa was possible only after terminal sialic acid residues were removed (Peláez and Long, 2007). In particular, lectins GS-II and SBA (corresponding to specific configurations of *N*-acetyl-glucosamine and *N*-acetylgalactosamine residues) were not visualized on turkey spermatozoa prior to neuraminidase treatment but were localized to the acrosome and acrosome/head regions, respectively, upon removal of terminal sialic acid residues. Given the regional localization of these carbohydrates, it seems plausible that the particular glycoconjugates apparently masked by sialic acid have an important functional role in fertilization. It is important to note that the observed alterations in carbohydrate abundance over time in the current study likely were not a result of sialic acid residues being lost and/or cleaved during in vitro semen storage. The main evidence for this assertion is that levels of sialic acid on the sperm surface did not decrease until the 24-hour time point, whereas increased abundance of some carbohydrate groups occurred as early as 2 hours after the start of semen storage.

Of particular interest is the finding that lectins differentially bound to spermatozoa from males exhibiting either the high- or low-mobility phenotype, which indicates that the glycocalyx composition of these 2 phenotypes is dissimilar and is suggestive of functional implications for both fresh and in vitro stored semen. It has been shown for both turkeys and chickens that males characterized with the high-mobility phenotype provide superior fertility rates compared with those of low-mobility males (Donoghue et al, 1998; Birkhead et al, 1999). In fact, mobility is the only sperm attribute shown to be directly correlated with fertility outcome in poultry (Froman et al, 1999; King et al, 2000). In chickens, mobility phenotype is determined by mitochondrial function (Froman and Kirby, 2005), which depends on the extent of intracellular Ca^{2+} uptake (Froman et al, 2006). Although a similar mechanism is likely for turkey mobility phenotype, our findings implicate additional distinguishing factors at the molecular level of the sperm plasma membrane. In our study, when differences were found between mobility phenotypes with respect to lectin binding, the MnFI values were consistently higher in the low-mobility males, indicating a higher content of the specific carbohydrate. Interestingly, the increased abundance of some mannose/glucose, *N*-acetyl-glucosamine, and *N*-acetylgalactosamine glycoconjugates in the plasma membrane of spermatozoa from low-mobility males was evident in fresh as well as in vitro stored semen. In mammals, differences in plasma membrane glycoconjugates have

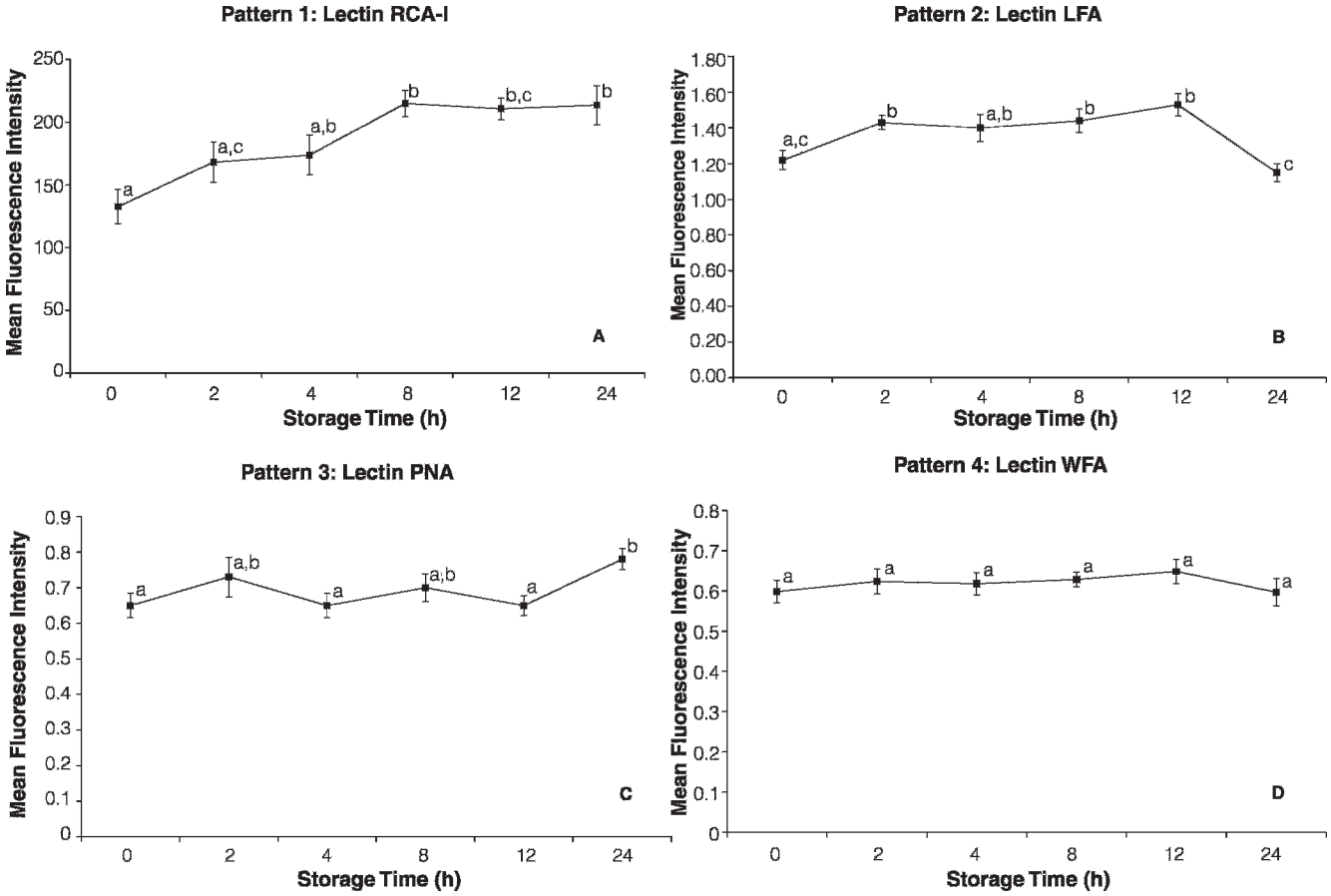

Figure 1. Patterns of changes in mean fluorescence intensity (MnFI; mean ± SEM) over time during storage of turkey semen at 4°C (n = 6 replicates). **(A)** Increased MnFI that remained elevated for the remaining time points; **(B)** increased MnFI that peaked at specific time points and subsequently decreased during the remaining time points; **(C)** baseline MnFI until the 24-hour time point; and **(D)** no significant changes in MnFI during 24-hour storage. Means with different superscripts were significantly different ($P < .05$). Lectin RCA-I indicates *Ricinus communis* lectin-I; Lectin LFA, *Limax flavus* lectin; Lectin PNA, *Arachis hypogaea* lectin; Lectin WFA, *Wisteria floribunda* lectin.

been found in spermatozoa exhibiting different rates of motility (González-Chábarri et al, 1994), thereby supporting the hypothesis that glycocalyx characteristics have physiological significance with respect to poultry sperm mobility phenotype.

In the light of these findings, the major question arising from the current study is the functional significance of the quantitative changes described. Carbohydrates of biological fluids and cell surfaces play a crucial role in reproduction. Evidence is accumulating that carbohydrate groups on the sperm surface regulate early gamete interactions (Diekman, 2003). The role of specific sperm surface glycoproteins in the process of fertilization and sperm physiological events, such as capacitation and the acrosome reaction, has been reported for a wide range of species and taxa (Kopecny and Flechon, 1987; Veselsky et al, 1992; Bérubé and Sullivan, 1994; Lassalle and Testart, 1996; Evans, 1999; Yu et al, 2002; Srivastav et al, 2004). Changes in the sperm glycocalyx, such as the molecular remodeling of plasma membrane components during capacitation of mammalian sperm, subsequently affect sperm function (Eddy, 1988). In particular, glycocalyx modifications occurring during mammalian spermatozoa maturation in the epididymis are believed to confer fertilizing ability to the spermatozoon (Eddy, 1988). Moreover, the characteristics of changes occurring in the glycocalyx of boar spermatozoa during capacitation and acrosome reaction have implications for fertility (Jiménez et al, 2002). Based on the available literature, it seems reasonable to suggest that the modifications of the turkey sperm glycocalyx observed in our study could have functional repercussions that affect the fertility of in vitro stored semen. We also can speculate that known differences in fertility between high- and low-mobility phenotype males may be mediated, in part, through differences in glycocalyx composition. For example, the levels of *N*-acetyl-galactosamine clearly differed between

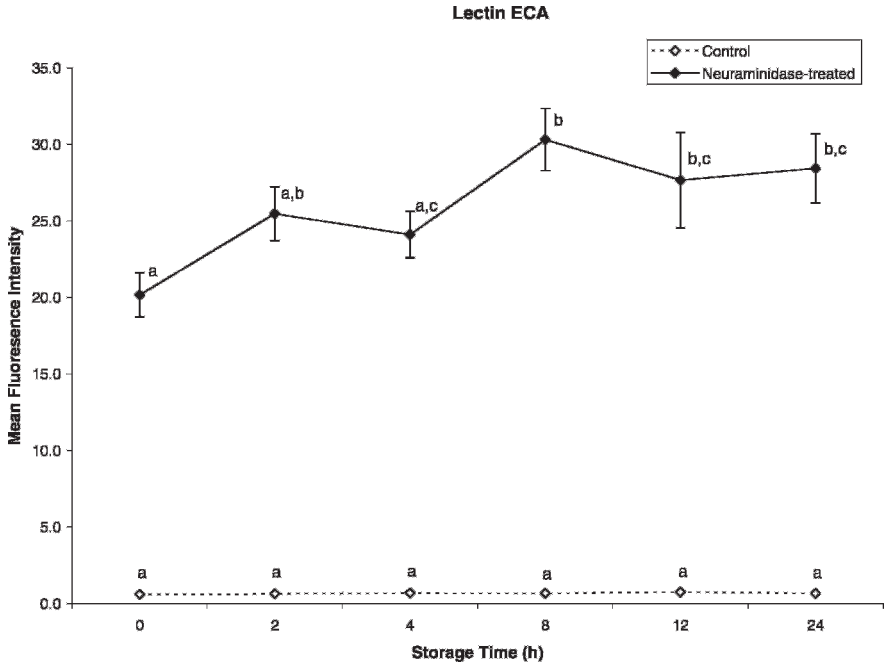

Figure 2. Representative *Erythrina cristagalli* lectin (Lectin ECA) exhibiting differing mean fluorescence intensity patterns between control and neuraminidase-treated spermatozoa. Within treatment, means with different superscripts were significantly different ($P < .05$).

spermatozoa from high- and low-mobility phenotype males, suggesting an important functional role for this carbohydrate and associated glycoconjugate(s). We are planning studies to identify the glycoconjugates that were altered during in vitro storage of turkey semen to determine the functional significance of these poststorage carbohydrate modifications and increase our current knowledge on how storage protocols affect the fertility of turkey spermatozoa.

Until more is known regarding the relationship between alterations of glycocalyx and functionality of spermatozoa in poultry species, the experimental data reported here permit only speculation about the potential negative impact of an increased proportion of nonsialylated sugar residues on the fertility of semen. The increased MnFI values observed for most lectins during storage indicate that more binding occurs as a result of changes in the availability of sugar residues over time. Because sialic acid residues do not decrease during incubation, the observed increases in lectin binding probably do not result from a phenomenon of shedding this terminal sugar and unmasking of subterminal residues. Rather, new components could be incorporated into the glycocalyx from free carbohydrates existing in the seminal plasma, and/or enzymatic transformations could operate in the existing sugar moieties over the incubation period. The seminal plasma of poultry species is known to contain free sugar residues as well as the specific enzymes (glycosidases, glycosyl transferases) for driving these processes (Ahluwalia and Graham, 1966; Droba and Droba, 1987, 1992; Droba and Dzugan, 1993). The net increase in unmasked sugar residues would increase the antigenicity of spermatozoa and would have negative consequences for the storage of sperm in the female reproductive tract (sperm storage tubules) because sperm selection in the vagina of poultry species is based on immunologic recognition of surface antigenicity (Steele and Wishart, 1992), and proper masking of sugar residues by sialic acid is essential for ensuring the transvaginal migration of spermatozoa (Steele and Wishart, 1996). In the chicken, it has been demonstrated that artificially increasing the antigenicity of sperm results in fewer sperm reaching the storage tubules and a lower proportion of fertile eggs (Froman and Thursam, 1994). If a similar sialic acid selection mechanism applies to the turkey, then increased antigenicity as a result of cold semen storage would negatively impact fertility. Further research is needed to verify whether incorporation/transformation of glycocalyx sugar residues occurs as a result of the availability of free carbohydrates and/or enzymes from the seminal plasma during storage of turkey semen. If so, improvements in the storage protocol such as removal of seminal plasma or addition of molar concentrations of sialic acid to the extender medium could be proposed.

In conclusion, quantitative changes occur in the carbohydrate content of turkey sperm surface glycoca-

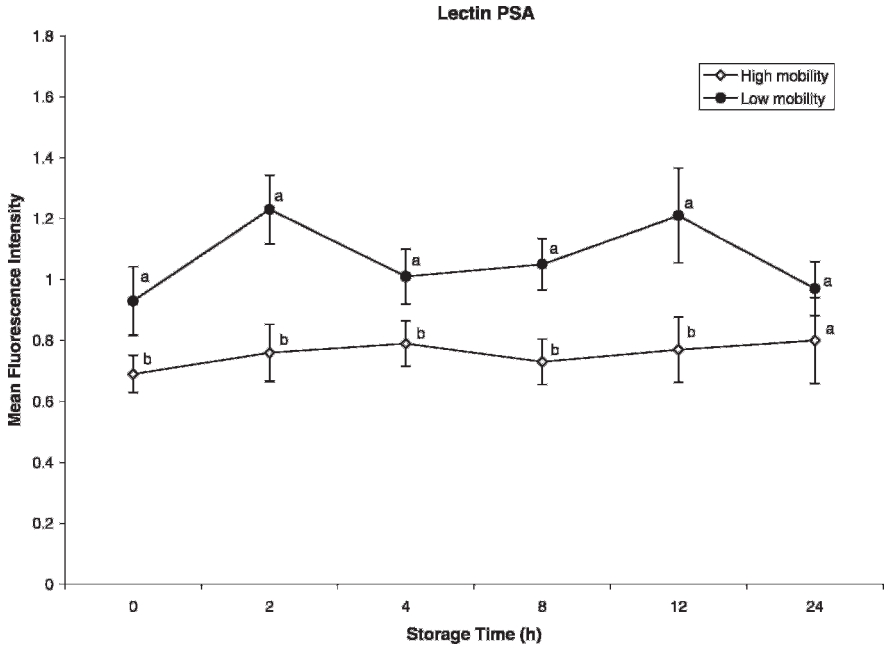

Figure 3. Effect of the sperm mobility phenotype on the mean fluorescence intensity (MnFI; mean ± SEM) of stored turkey spermatozoa incubated with *Pisum sativum* lectin (Lectin PSA; n = 6 replicates). MnFI values were similar ($P > .05$) over time for both high- and low-mobility semen but differed between mobility groups throughout storage except the 24-hour time point (means with different superscripts were significantly different [$P < .05$]).

lyx during a 24-hour period of storage at 4°C. The pattern of changes varies among sugar residues, with increased rates of lectin binding being observed during the incubation period. Of special interest is the fact that sperm glycocalyx is not considerably altered until after 6 hours of storage. Sperm of low-semen–mobility phenotype appear to contain higher amounts of some sugar moieties compared with the high-mobility phenotype and also present differences in the pattern of changes with regard to the latter. Increases in the rate of lectin binding reveal an augmentation of nonsialylated terminal residues that could modify sperm antigenicity, eventually impacting fertility negatively. Other potential functional implications of the carbohydrate modifications reported here are not known and warrant investigation. The mechanism responsible for changes is also a clear area for further research. Previous work on the effects of cold storage on turkey sperm plasma membrane has mainly concerned the lipid component (Douard et al, 2000, 2003, 2005). To the authors' knowledge, this is the first report that other important functional constituents are also affected.

Acknowledgments

We thank W. Smoot for assistance in semen collection, as well as T. Conn and G. Welch for valuable expertise in laboratory management and flow cytometry analysis. The sponsorship provided by the Office of International Relations of the Smithsonian Institution for a postdoctoral stay of J. Peláez is greatly acknowledged.

References

Ahluwalia BS, Graham EF. Free carbohydrates in seminal plasma and sperm cells of semen of fowl. *J Reprod Fertil.* 1966;12:359–361.

Allen T, ed. *AWIC (Animal Welfare Institutional Committee) Resources Series No.7.* Beltsville, Md: US Department of Agriculture; 2000.

Bérubé B, Sullivan R. Inhibition of in vitro fertilization by active immunization of male hamsters against a 26-kDa sperm glycoprotein. *Biol Reprod.* 1994;51:1255–1263.

Birkhead TR, Martinez JG, Burke T, Froman DP. Sperm mobility determines the outcome of sperm competition in the domestic fowl. *Proc R Soc London B.* 1999;266:1759–1764.

Burrows WH, Quinn JP. The collection of spermatozoa from the domestic fowl and turkey. *Poult Sci.* 1937;14:251–254.

Carrasco JL, ed. *The Statistical Method in Medical Research* [in Spanish]. Madrid, Spain: Ciencia 3; 1995.

Diekman AB. Glycoconjugates in sperm function and gamete interactions: how much sugar does it take to sweet-talk the egg? *Cell Mol Life Sci.* 2003;60:298–308.

Donoghue AM, Holsberger DR, Evenson DP, Froman DP. Semen donor selection by in vitro sperm mobility increases fertility and semen storage in the turkey hen. *J Androl.* 1998;19:295–301.

Donoghue AM, Wishart GJ. Storage of poultry semen. *Anim Reprod Sci.* 2000;62:213–232.

Douard V, Hermier D, Blesbois E. Changes in turkey semen lipids during liquid in vitro storage. *Biol Reprod.* 2000;63:1450–1456.

Douard V, Hermier D, Labbe C, Magistrini M, Blesbois E. Role of seminal plasma in damage to turkey spermatozoa during in vitro storage. *Theriogenology*. 2005;63:126–137.

Douard V, Hermier D, Magistrini M, Blesbois E. Reproductive period affects lipid composition and quality of fresh and stored spermatozoa in turkeys. *Theriogenology*. 2003;59:753–764.

Droba M, Droba B. Purification and properties of beta-galactosidase from chicken seminal plasma. *Comp Biochem Physiol B*. 1987;87:379–384.

Droba B, Droba M. Properties of acid beta-*N*-acetyl-D-glucosaminidase from cock semen. *Folia Biol (Krakow)*. 1992;40:67–71.

Droba B, Dzugan M. Acid alpha-D-mannosidase from turkey seminal plasma. *Folia Biol (Krakow)*. 1993;41:49–54.

Eddy EM. The spermatozoon. In: Knobil E, Neill JD, Ewing LL, Market CL, Greenwald GS, Ptaff DW, eds. *The Physiology of Reproduction.* Vol 1. New York, NY: Raven Press; 1988: 27–68.

Etches RJ, ed. *Reproduction in Poultry*. Oxfordshire, United Kingdom: CAB International; 1996.

Evans JP. Sperm disintegrins, egg integrins, and other cell adhesion molecules of mammalian gamete plasma membrane interactions. *Frontiers Biosci*. 1999;4:114–131.

Froman DP, Engel HN. Alteration of the spermatozoal glycocalyx and its effect on duration of fertility in the fowl (*Gallus domesticus*). *Biol Reprod*. 1989;40:615–621.

Froman DP, Feltmann AJ. Sperm mobility: a quantitative trait of the domestic fowl (*Gallus domesticus*). *Biol Reprod*. 1998;58:379–384.

Froman DP, Feltmann AJ, Rhoads ML, Kirby JD. Sperm mobility: a primary determinant of fertility in the domestic fowl (*Gallus domesticus*). *Biol Reprod*. 1999;61:400–405.

Froman DP, Kirby JD. Sperm mobility: phenotype in roosters (*Gallus domesticus*) determined by mitochondrial function. *Biol Reprod*. 2005;72:562–567.

Froman DP, Thursam KA. Desialylation of the rooster sperm's glycocalyx decreases sperm sequestration following intravaginal insemination of the hen. *Biol Reprod*. 1994;50:1094–1099.

Froman DP, Wardell JC, Feltmann AJ. Sperm mobility: deduction of a model explaining phenotypic variation in roosters (*Gallus domesticus*). *Biol Reprod*. 2006;74:487–491.

González-Chábarri E, Aguado JA, Navarro M, López-Rodas V, Costas E. The use of lectins to characterize and separate living canine spermatozoa: a preliminary study. *Theriogenology*. 1994;41: 365–369.

Jiménez I, González-Márquez H, Ortiz R, Betancourt M, Herrera J, Fierro R. Expression of lectin receptors on the membrane surface of sperm of fertile and subfertile boars by flor cytometry. *Arch Androl*. 2002;48:159–166.

King LM, Kirby JD, Froman DP, Sonstegard TS, Harry DE, Darden JR, Marini PJ, Walker RM, Rhoads ML, Donoghue AM. Efficacy of sperm mobility assessment in commercial flocks and the relationships of sperm mobility and insemination dose with fertility in turkeys. *Poult Sci*. 2000;79:1797–1802.

Kopecny V, Flechon JE. Ultrastructural localization of labeled acrosomal glycoproteins during in vivo fertilization in the rabbit. *Gamete Res*. 1987;17:35–42.

Lassalle B, Testart J. Lectins binding on human sperm surface increase membrane permeability and stimulate acrosomal exocytosis. *Mol Hum Reprod*. 1996;2:651–658.

Morris SA, Howarth B Jr, Crim JW, Rodriguez de Cordoba A, Esponda P, Bedford JM. Specificity of sperm-binding Wolffian duct proteins in the rooster and their persistence on spermatozoa in the female host glands. *J Exp Zool*. 1987;242:189–198.

Peláez J, Long JA. Characterizing the glycocalyx of poultry spermatozoa: I. Identification and distribution of carbohydrate residues using flow cytometry and epifluorescence microscopy. *J Androl*. 2007;28:342–352.

Robertson L, Wishart GJ, Horrocks AJ. Identification of perivitelline N-linked glycans as mediators of sperm-egg interaction in chickens. *J Reprod Fertil*. 2000;120:397–403.

Srivastav A, Singh B, Chandra A, Jamal F, Khan MY, Chowdhury SR. Partial characterization, sperm association and significance of *N*- and *O*-linked glycoproteins in epididymal fluid of rhesus monkeys (*Macaca mulatta*). *Reproduction*. 2004;127:343–357.

Steele MG, Wishart GJ. Demonstration that the removal of sialic acid from the surface of chicken spermatozoa impedes their transvaginal migration. *Theriogenology*. 1996;46:1037–1044.

Steele MG, Wishart GJ. Evidence for a species-specific barrier to sperm transport within the vagina of the chicken hen. *Theriogenology*. 1992;38:1107–1114.

Veselsky L, Jonakova V, Sanz ML, Topfer-Petersen E, Cechova D. Binding of a 15 kDa glycoprotein from spermatozoa of boars to surface of zona pellucida and cumulus oophorus cells. *J Reprod Fertil*. 1992;96:593–602.

Yu S, Kojima N, Hakomori S, Kudo S, Inoue S, Inoue Y. Binding of rainbow trout sperm to egg is mediated by strong carbohydrate-to-carbohydrate interaction between (KDN)GM3 (deaminated neuraminyl ganglioside) and Gg3-like epitope. *Proc Natl Acad Sci U S A*. 2002;99:2854–2859.